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Note

Simplified buffer system for accelerated column analysis of amino acids in physiological fluids

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The separation and determination of amino acids in biological fluids by elution chromatography on ion-exchange columns has developed considerably since the introduction of the first cation-exchange resin by Moore and Stein [1]. The method acquired great importance after its automation by Spackman and Moore [2] and later through the introduction of lithium citrate rather than sodium citrate buffers [3, 4], thus allowing the resolution of asparagine, glutamic acid and glutamine.

The most commonly used resins are made from sulphonated polystyrene crosslinked with 8—12% of divinylbenzene. They are characterized by their good stability but low reactivity, which necessitates the use of a high operating temperature; this in turn causes a loss of resolution for some amino acids. Hamilton et al. [5] showed the relationships between resin-bead diameter, buffer flow-rate and column length and diameter. The use of increasingly fine resin beads permits the operating temperature to be decreased, and also the column length and diameter [6], while maintaining the number of buffer plateaus needed for the fractionation of complex mixtures. This results in greater sensitivity and speed of analysis.

In the most recently described techniques, either a single column with a continuous buffer gradient or two columns with stepwise buffer changes are used. In the first instance, it is difficult to obtain satisfactory chromatogram reproducibility and, as Dautrevaux [7] pointed out, the identification of peaks is difficult, especially for biological fluids such as urine. In the second instance, two portions of the same sample must be used and the analysis takes 6 h.

As techniques improve, the demand for amino acid determinations constantly increase: for the diagnosis of aminoacidopathy resulting from a catabolic deficit; for the search of heterozygotes by tolerance tests; and for dietary surveillance of certain treated aminoacidopathies.

We describe here a modification of the Technicon technique [8] for the NC 11 P AutoAnalyzer. Koehl and Mandel [9] had already modified this technique by programming an automatic temperature change for the columns and by running two columns in parallel, thus doubling the capacity of the instrument and permitting simultaneous analyses of two different samples. Our modification substitutes three buffers for the seven previously used, and allows the separation of 40 amino acids and ninhydrin-positive substances of interest in human biology from as little as 50 μl of plasma or urine.

MATERIALS AND METHODS

Materials

Two columns (470 × 5 mm) mounted in parallel and fed by two high-pressure pumps, which are temperature-controlled by two Haake water-baths, are each packed with a 41-cm bed of Chromobeads C_3 resin beads (particle diameter 10 μm). These columns are linked to the NC 11 P Technicon system, consisting of a P 111 pump, a peristaltic valve and a programmer, a 95° water-bath with a double coil, two single-channel colorimeters for the AutoAnalyzer 11 equipped with a set of interference filters at 410 nm, and a double-pen recorder.

Reagents

The ninhydrin reagents are prepared as recommended by Technicon, ninhydrin and hydrazine sulphate being mixed as required. The pH of the ninhydrin reagent is adjusted to 6.30 for increased sensitivity [10].

After they have been used, the resins are regenerated in 0.3 *M* lithium hydroxide solution and re-equilibrated with the first buffer at pH 2.75.

The lithium citrate elution buffers [3, 11, 12] are prepared as shown in Table I.

Sample preparation

Plasma or urine samples are deproteinized with 5% or 2% sulphosalicylic acid, respectively. They are then diluted with an equal volume of the sample-dilution (pH 2.20) described by Benson et al. [3]. The final pH of the sample is 2.20.

Standard mixture

The amino acids in the standard mixture have concentrations of 2.5 μmoles/ml in 0.1 *M* hydrochloric acid, except β-alanine, sarcosine, β-aminoisobutyric acid and urea (10 μmoles/ml).

Methods

The flow diagram is shown in Fig. 1, and Table II gives the detailed programme of the peristaltic valve settings, showing all of the conditions for chromatographic elution.

Four positions (numbers 5—8) are used for transit of buffers and lithium hydroxide. The first buffer change takes place 96 min after the beginning of

TABLE I

BUFFER COMPOSITIONS

		Stock solution
Lithium concentration	(*M*)	3
Citrate concentration	(*M*)	0.5
Lithium tricitrate. $4H_2O$	(g)	150.5
Lithium chloride	(g)	59.05
Thiodiglycol	(ml)	25
Caprylic acid	(ml)	1
30% Brij-35	(ml)	20
Distilled water		to 1 l

		Buffer 1	Buffer 2	Buffer 3
Stock solution	(ml)	100	100	400
Methyl Cellosolve	(ml)	35	–	–
Distilled water		to 1 l	to 1 l	to 1 l
Final pH at 25°		2.75	3.50	4.10

the run, and the second 46 min later. The flow-rate is set at 0.47 ml/min. The pressure does not exceed 600 p.s.i. at the lower temperature and decreases to 400 p.s.i. at the higher temperature.

Two positions (numbers 21 and 22) are used for ninhydrin or methyl Cellosolve, the flow-rate of which is set at 0.8 ml/min.

Four positions (numbers 13–16) are used to change the temperature of the columns automatically. For the first 180 min, the effluent from the first water-bath holds both columns at 37° by means of peristaltic valve; meanwhile the water in the second water-bath does not circulate. Then the circuit switches to the second water-bath, raising the column temperature to 55° [13] and blocks the effluent from the first one.

This temperature programme, derived from that described by Koehl and Mandel [9], is very effective because the temperature change is effected very rapidly. We have not found any serious disadvantage in blocking the circulation of the unused water-bath.

After completion of the runs, the detection circuit is rinsed with a 50% solution of methyl Cellosolve in distilled water, replacing the flow from the column (positions 17–20) in order to maintain a constant flow-rate in the reaction water-bath and the colorimeter, while the column is regenerated and the samples are changed. Meanwhile, the regeneration eluate is by-passed into the drain (positions 2 and 4) without passing through the colorimeter.

Two chromatograms can be obtained in 5 h. It then takes 45 min to regenerate and re-equilibrate the columns and rinse the circuit, after which two new samples can be applied.

RESULTS AND DISCUSSION

Fig. 2 shows the results obtained from a standard solution of 40 amino acids

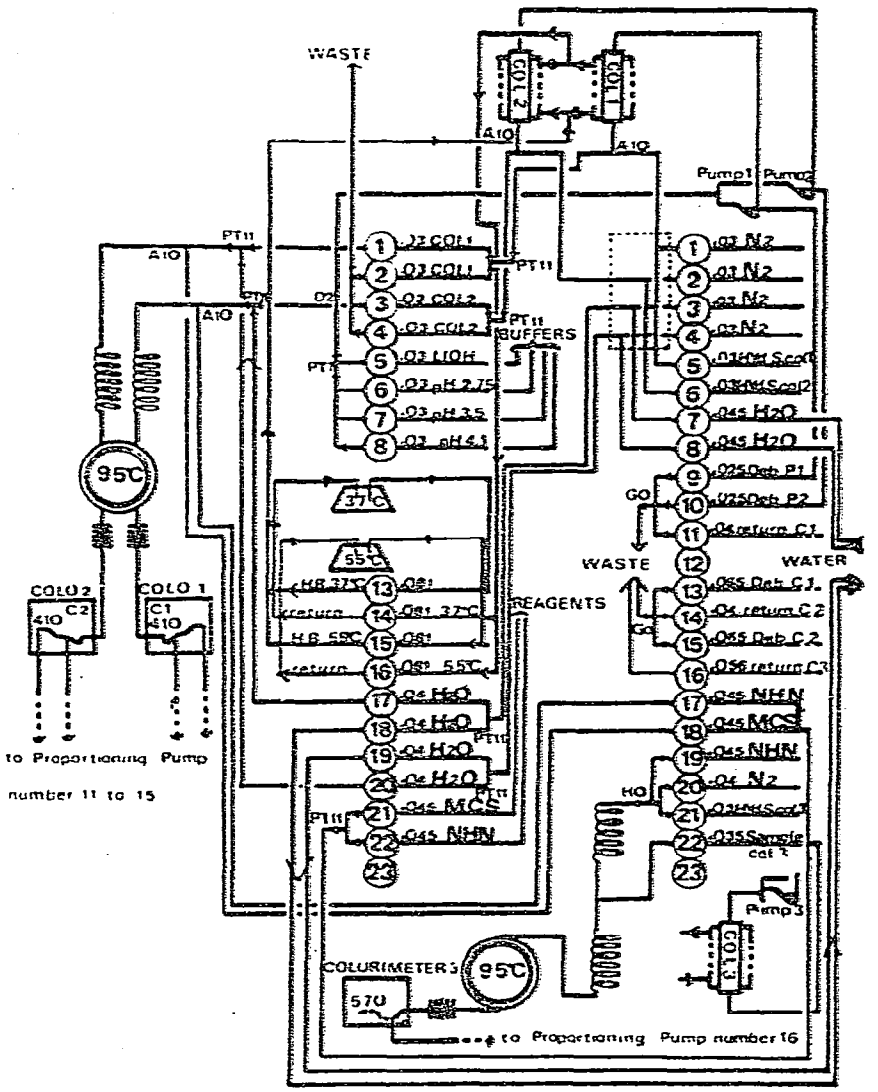

Fig. 1. Flow diagram.

and related substances and from biological samples. Chromatograms are shown for simultaneous runs on the plasma and urine from a child suffering from a liver disease and suspected to have tyrosinosis. In Table III we list the amino acids separated with each buffer under the described conditions of time, pH and temperature.

The first buffer (pH 2.75) elutes amino acids up to valine. However, phosphoserine and cysteinesulphinic acid are not resolved from cysteic acid. The sharpness in resolution of the amino acids from aspartic acid to citrulline de-

TABLE II

PROGRAMME OF THE NC II P PERISTALTIC VALVE FOR PHYSIOLOGICAL FLUIDS

L = large diameter space, S = small diameter space.

Running time (min)			2	49	96	122	142	182	222	262	302	310	330	346
Roller time (min)			2	47	47	26	20	40	40	40	40	8	20	16
Grip-ring washer			L	L	L	L	L	L	L	L	L	L	L	L
Analytical system Column 1		1		S	S	S	S	S	S	S	S	S		
Waste column 1		2	S										S	S
Analytical system Column 2		3		S	S	S	S	S	S	S	S	S		
Waste column 2		4	S										S	S
0.30 *M* LiOH		5										S		
Buffer, pH 2.75		6	S	S	S								S	S
Buffer, pH 3.50		7				S	S							
Buffer, pH 4.10		8						S	S	S	S			
Open		12			FIXED SPACER									
Water-bath 37°	Inlet columns	13	S	S	S	S	S	S						
	outlet columns	14	S	S	S	S	S	S						
Water-bath 55°	Inlet columns	15							S	S	S	S	S	S
	Outlet columns	16							S	S	S	S	S	S
Water system 1	Analytical circuit	17	S										S	S
	Waste	18		S	S	S	S	S	S	S	S	S		
Water system 2	Waste	19		S	S	S	S	S	S	S	S	S		
	Analytical circuit	20	S										S	S
Methyl Cellosolve (50%)		21	S										S	S
Ninhydrin		22		S	S	S	S	S	S	S	S	S		
Pump 1			S	S	S	S		S	S	S	S	S	S	S
Pump 2			S	S	S	S	S	S	S	S	S	S	S	S
Roller numbers			1	2	3	4	5	6	7	8	9	10	11	12

pends both on the precision of the buffer pH to ± 0.01 unit, and on the exact percentage of methyl Cellosolve: if the latter is below 3.5%, proline is not resolved from glycine; if it is above 3.5%, proline overlaps with glutamine, and asparagine is not resolved from glutamic acid.

The second buffer (pH 3.50) elutes amino acids up to tyrosine.

The change to the third buffer (pH 4.10) takes place during the elution of the leucine peak, 142 min after the beginning of the run. Maintaining the column temperature at 37° for the next 40 min gives a good resolution of tyro-

TABLE III

AMINO ACIDS OR COMPOUNDS ELUTED AS A FUNCTION OF THE THREE BUFFERS USED AND THE TEMPERATURE

Cysteic acid Taurine Phosphoethanolamine Urea Aspartic acid Hydroxyproline Methionine sulphone Threonine Serine Asparagine Glutamic acid Glutamine α-Aminoadipic acid Sarcosine Proline Glycine Alanine Citrulline α-Aminobutyric acid Valine	0.3 *M* Li buffer pH 2.75 + 3.5% methyl Cellosolve 96 min	37° 182 min
Cystine Cystathionine Methionine Isoleucine Leucine Norleucine	0.3 *M* Li buffer pH 3.50 46 min	
Tyrosine β-Alanine Phenylalanine Homocystine γ-Aminobutyric acid	1.2 *M* Li buffer pH 4.10 40 min	
Ethanolamine Ammonia Ornithine Lysine Histidine 1-Methylhistidine 3-Methylhistidine Arginine	120 min	55° 120 min

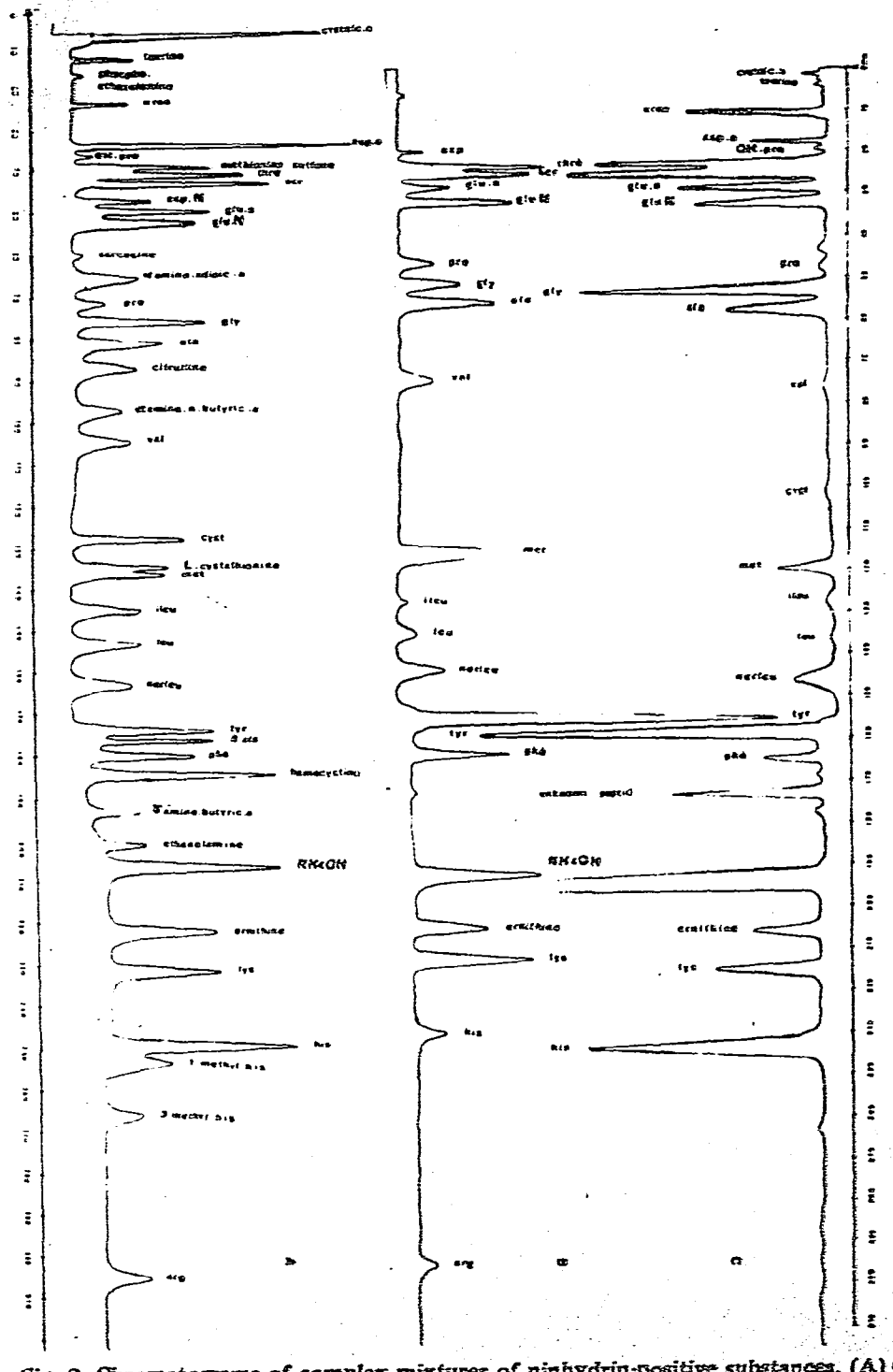

Fig. 2. Chromatograms of complex mixtures of ninhydrin-positive substances. (A) Chromatogram of a standard solution containing 0.2 μmoles of urea, sarcosine, and β-alanine, and 0.05 μmoles of the other compounds; (B) elution of 50 μl of a patient's plasma; and (C) simultaneous elution of 100 μl of urine from the same patient.

sine, β-alanine, phenylalanine, homocysteine and γ-aminobutyric acid. Only β-aminoisobutyric acid is not resolved from phenylalanine under these conditions. However, an excess of phenylalanine can be confirmed by simultaneous fluorimetric assay. Furthermore, β-aminoisobutyric acid is rare in biological fluids. Finally, if the pH of the pH 4.10 buffer is changed by 0.02 unit, β-aminoisobutyric acid is then separated from phenylalanine, but at the expense of β-alanine. Increasing the column temperature to 55° as the ammonia peak emerges allows the separation of the basic amino acids in 2 h.

The rapid preparation of the reagents and low sample volume (50 μl of plasma) required for a run permit micro-sampling. In addition, the daily output of the apparatus can be increased by using an automatic sample injector.

The system described here permits simultaneous dietary surveillance of treatment for leucinosis and phenylketonuria by assay on a 300 × 6 mm column packed with C_2 Chromabeads (13-μm particles). Small groups of amino acids such as valine, isoleucine and leucine can be resolved within 75 min and tyrosine and phenylalanine within 45 min [14]. As these partial chromatographic analyses are made independently of the system just described, the daily output of complete analyses is not reduced.

CONCLUSION

The use of three successive buffers for the simultaneous chromatography of two biological samples in 5½ h permits a good resolution and a good reproducibility of the results: variations in retention times are very small. Because the buffer changes, the temperature changes, the regeneration of the columns and the switching off are automated, up to six samples per day can be analyzed.

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